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(54) Method for stabilizing immunologically active substances immobilized on an insoluble carrier and their use in the preparation of reagents for measuring physiologically active substances.

(57) An immuno active substance immobilized on a carrier and stabilized by immersing said carrier in a solution of at least one of sugars and proteins can be used for measuring a physiologically active substance even after stored for a long period of time.

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*immobilizing  
fixing agents to solid  
support  
(minwell)*

## STABILIZING METHOD OF IMMUNO ACTIVE SUBSTANCE

## IMMOBILIZED ON INSOLUBLE CARRIER AND

## ITS USE IN PREPARATION OF REAGENT

## FOR MEASURING PHYSIOLOGICALLY ACTIVE SUBSTANCE

1           This invention relates to a process for stabilizing  
immuno active substances immobilized on an insoluble carrier  
and preparation of reagents for measuring a physiologically  
active substance utilizing the immuno active substances  
5 stabilized by the above process as their components.


Antigen-antibody reactions have been used for  
measuring or detecting various physiologically active sub-  
stances due to their high specificity and high sensitivity.  
Specifically, radioimmunoassay (hereinafter referred to as  
10 "RIA") systems have been applied to measure trace substances  
(e.g., hormones such as insulin, glucagon, thyroxine, etc.;  
high-molecular weight physiologically active substances  
such as immunoglobulin E (Ig E),  $\alpha$ -fetoprotein, CEA (carcino-  
embrionic antigen), etc.) in biological samples such as  
15 serum, urine, and tissue fluid, since RIA particularly  
allows highly sensitive measurement. But it is also true  
that the prevalence of RIA is limited due to some disadvant-  
age. Reagents used in RIA are expensive and often unstable.  
Complicated and expensive apparatuses are required for  
20 reading the any results. Most of all, special safety stand-  
ards are required to be get for handling of radioisotopes  
and disposal of radioactive wastes.

On the other hand, the enzyme immunoassay (herein-  
after referred to as EIA) was introduced in 1971 in order to  
25 overcome these disadvantages in RIA. In EIA, an enzyme

1 is used as labeling substance instead of radioactive isotope.  
An enzyme labeled reagent is inexpensive and stable for  
a long period of time. EIA has the equivalent or higher  
sensitivity for measurement as RIA. Further the test results  
5 can be measured by the naked eye or a simple apparatus. Due  
to such advantages over RIA, application of EIA is rapidly  
expanded. But RIA and EIA are based on the same measuring  
principles and only differ in their labeling substances.  
As to measuring systems, there have been reported various  
10 kinds of measuring systems, which can be divided into two  
groups, that is, the heterogeneous measuring system which  
employs the B/F separating method and the homogeneous measur-  
ing system which does not employ the B/F separating method.  
The B/F separating method indicates that a bound form of  
15 an antigen and an antibody as a result of antigen-antibody  
reaction (bound type, B) and a free form of an antigen and  
antibody (free type, F) are physically separated. Homogene-  
ous system depends on inhibition or activation of the enzyme  
by one of the components (mainly antibody) after antigen-  
20 antibody reaction. Since few cases of such enzyme-hapten  
complex have been reported, the application of the homogene-  
ous system is limited. Therefore, most present RIA and EIA  
employ the heterogeneous measuring system. In the heterogene-  
ous measuring system, a solid phase method wherein an antigen  
25 or an antibody is immobilized on a water-insoluble carrier  
has been most frequently employed for the B/F separation.  
Although natural high-molecular compounds such as cellulose,  
Sephacrose, agarose and dextran have been used as the water-

1 insoluble carrier, these compounds require much time for  
washing procedure and centrifugational procedure, which  
results in becoming a major factor for causing scattering  
of measured values. In order to overcome these problems,  
5 inorganic materials such as glass and synthetic polymers  
such as polystyrene, polypropylene, poly(vinyl chloride)  
are recently used as a carrier in the form of tubes, beads,  
disks, fine particles (latex particles), microplates. By  
using these materials as carrier, the centrifugational  
10 procedure becomes unnecessary, and the washing procedure can  
be simplified remarkably. Thus, reproducibility of measured  
values becomes good, and employment of automated system  
becomes possible and is actually practiced in some assay  
fields. By the reasons mentioned above, establishment of  
15 useful assay system in the heterogeneous measuring system  
using the solid phase method much depends on a quality of  
carrier.

Preferable properties of the carrier are as  
follows:

- 20 (1) When an immuno active substance is bound to the  
carrier, it should retain the immunological activity.
- (2) The carrier has no non-specific adsorption of  
components included in a test sample.
- (3) The carrier has properties of binding strongly  
25 with an immuno active substance.
- (4) The carrier has such properties as a surface  
structure which makes binding with a sufficient amount of  
immuno active substance possible.
- 

1       (5)       Handling such as washing procedure accompanied  
in the B/F separating procedure is simple and easy.

          In order to prepare a carrier which satisfies  
the properties mentioned above and on which an immuno active  
5   substance is attached, not only the selection of kind of  
carrier but also the binding method of immuno active sub-  
stance and the storing method of the carrier bound substance  
are subject matters for development. In RIA and EIA employ-  
ing the solid phase method, an immuno active substance is  
10   immobilized on a carrier such as glass beads, polystyrene  
beads, by covalent bond or physical adsorption method, and  
the carrier bound substance is stored in a buffer solution  
containing serum albumin.

          But such a method has many problems in that (i)  
15   it is necessary to extract the buffer solution using  
a filter paper at the time of use, which results in requiring  
much labor and causing scattering of measured values, (ii)  
when dried, deterioration of the solid phase takes place  
due to decrease of the immunological activity of immuno  
20   active substance, and (iii) there are many technical  
problems for designing an automated assay system.

          It is an object of this invention to provide a  
process for stabilizing an immuno active substance immobiliz-  
ed on a carrier overcoming the disadvantages mentioned above,  
25   and to provide a reagent utilizing the immuno active  
substance thus stabilized on a carrier as its component  
for measuring physiologically active substances.

          This invention provides a process for stabilizing

1 an immuno active substance immobilized on a carrier, which  
comprises immersing a carrier bound an immuno active  
substance in a solution of at least one member selected  
from the group consisting of a sugar and a protein.

5 This invention also provides an assay reagent  
for measuring a physiologically active substance comprising  
as a component an immuno active substance immobilized on  
a carrier and stabilized by immersing the carrier bound the  
immuno active substance in a solution of at least one member  
10 selected from the group consisting of a sugar and a protein.

As the carrier, there can be used any conventional  
insoluble ones usually used in RIA and EIA. Preferable  
carriers are insoluble (water-insoluble) ones which allow  
easy solid-liquid separation without conducting centrifuga-  
15 tional separation. Examples of such insoluble carriers  
are synthetic polymer compounds such as polystyrene, poly-  
propylene, poly(vinyl chloride), polyethylene, polychloro-  
carbonate, silicone resin, silicone rubber, etc.; inorganic  
materials such as porous glass, ground glass, alumina,  
20 silica gel, activated charcoal, metal oxides, etc. These  
materials can be used in any forms of tubes, beads, disk  
flakes, fine particles (latex particles), microplates, etc.

As a method for immobilizing the immuno active  
substance on the carrier, there can be used conventional  
25 methods such as a covalent coupling method and a physical  
adsorption method.

The covalent coupling method is a method for  
fixing an immuno active substance on a water-insoluble



1 carrier by covalent bond. The largest number of reports  
deal with this covalent coupling method among carrier binding  
methods. The functional groups which participate the  
binding of the immuno active substance with the carrier  
5 are an  $\alpha$ - or  $\epsilon$ -amino group, an  $\alpha$ -,  $\beta$ - or  $\gamma$ -carboxyl group,  
a sulfhydryl group, a hydroxyl group, an imidazole group,  
a phenol group, and the like. These functional groups react  
with a diazonium group, an acid azide, an isocyanate or  
an activated halogenated alkane. Therefore, by using such  
10 a reactive functional group, it becomes possible to bind the  
immuno active substance with the water-insoluble carrier by  
covalent bond (e.g. see Taisha vol. 8, page 696, 1971). In  
the case of using an inorganic material such as glass, the  
inorganic material is first treated with a trialkoxysilane  
15 derivative having a functional group such as  $\gamma$ -aminopropyl-  
triethoxysilane in order to introduce a reactive functional  
group thereinto. The resulting amino group-containing  
alkylated glass thus obtained can be bound with an immuno  
active substance by covalent bond by the same treatment as  
20 in the case of amino group-containing immuno active sub-  
stance. In general, introduction of a reactive aldehyde  
group by the treatment with glutaraldehyde has been widely  
used to couple an immuno active substance with a carrier  
(J. Biochem., vol. 80, p. 895, 1976). There can also be  
25 used various crosslinking agents depending on the kinds of  
immuno active substances. For example, there can be used  
succinaldehyde, malonaldehyde, or the like in addition to  
glutaraldehyde mentioned above for crosslinking an amino

1 group with an amino group, m-maleimidobenzoyl-N-hydroxy-  
succinimide ester, 4-(maleimidomethyl)cyclohexane-1-  
carboxyl-N-hydroxysuccinimide ester for crosslinking an  
amino group with a sulfhydryl group, and o-phenylenedi-  
5 maleimide for binding a sulfhydryl group with a sulfhydryl  
group.

The physical adsorption method is a method for  
immobilizing an immuno active substance on a water-insoluble  
carrier by physical adsorption. As the carrier, there can  
10 be used inorganic materials such as activated charcoal,  
porous glass, glass beads, alumina, a metal oxide, silica  
gel, hydroxy apatite, etc.; and synthetic polymer compounds  
such as polystyrene, polyethylene, poly(vinyl chloride),  
polypropylene, polychlorocarbonate, etc. Among them, the  
15 use of glass, polystyrene, or poly(vinyl chloride) in the  
form of tubes, beads, disk flakes, fine particles (latex  
particles), microplates are preferred.

As the immuno active substance to be immobilized  
on the carrier, there can be used an antigen, an antibody  
20 and a hapten (drugs, etc.).

Examples of the antigen are hormones such as  
insulin, glucagon, growth hormone, human chorionic  
gonadotropin, adrenocortical hormone, thyroid stimulating  
hormone, etc.; proteins such as IgG, IgM, IgA, IgE, IgD,  
25  $\alpha$ -fetoprotein, ferritin,  $\beta_2$ -microglobulin, CEA, etc.;  
and virus antigens such as HB<sub>s</sub> antigen, rubella virus  
antigen, etc.

Examples of the antibody are those obtained by





1 immunizing a mammal such as a rabbit, a guinea pig, a mouse,  
a goat, a sheep or the like, or a bird such as a chicken,  
a duck, or the like with an antigen or a hapten mentioned  
below by a conventional method (e.g., antiinsulin antibody,  
5 antiglucagon antibody, anti-IgG antibody, anti- $\alpha$ -fetoprotein  
antibody, anti- $\beta_2$ -microglobulin antibody, etc.).

Examples of the hapten are steroid hormones,  
catecholamines, and vitamins.

As the sugar solution, there can be used a solution  
10 obtained by dissolving a monosaccharide such as ribose,  
glucose, fructose, mannose, galactose, maltose, lactose,  
sucrose, or the like, an oligosaccharide, or a polysaccharide  
such as dextran, dextrin, or the like, these saccharides  
being used alone or as a mixture thereof, in purified water  
15 or a buffer solution. Among these sugar solutions, lactose,  
sucrose, and dextrin solutions are preferred.

As the protein solution, there can be used a  
solution obtained by dissolving a serum albumin such as a  
bovine serum albumin, a human serum albumin, a sheep serum  
20 albumin, or water-soluble gelatin, in purified water or a  
buffer solution. Among these protein solutions, bovine  
serum albumine and water-soluble gelatin solutions are  
preferred.

The sugar solution and the protein solution can  
25 be used alone or as a mixture thereof. When the mixed  
solution of sugar and protein is used, more excellent effects  
can be expected.

The sugar content in the sugar solution is

1 usually 0.1 to 10 weight/volume percent, preferably 2.5 to  
5 weight/volume percent.

The protein content in the protein solution is  
usually 0.1 to 2 weight/volume percent, preferably 0.5 to  
5 1.5 weight/volume percent.

When the solution contains both sugar and protein,  
the sugar content is usually 0.1 to 10 weight/volume percent,  
and preferably 2.5 to 5 weight/volume percent and the  
protein content is usually 0.1 to 2 weight/volume percent,  
10 and preferably 0.5 to 1.5 weight/volume percent.

As the solvent for dissolving a sugar and/or a  
protein, there can be used purified water or a buffer solu-  
tion. Examples of the buffer solution are those having  
buffering effect at near neutral pH such as a phosphate  
15 buffer solution, a tris-HCl buffer solution, a Good's buffer  
solution, and the like. Among them, the phosphate buffer  
solution is particularly preferred. The molar concentration  
of the buffer solution is usually 0.01 to 0.2 M, preferably  
0.02 to 0.05 M and the pH of it is preferably 6.8 to 7.2.

20 When preparing the solution of sugar and/or  
protein, there is no limitation to the order of addition  
of these materials.

In order to stabilize the immuno active material  
immobilized on a carrier in the dried state, the carrier  
25 attaching the immuno active material is first immersed in  
the solution of protein and/or sugar, for example, for 20  
to 40 minutes at room temperature, and dried, for example,  
by placing the thus treated carrier on a filter paper for

1 a sufficient time to allow air drying. The dried carrier  
with stabilized immuno active substance can be used as a  
reagent. More preferably, the thus dried carrier is  
stored in a vessel sealed and capped under nitrogen gas  
5 or reduced pressure. By subjecting the carrier to immers-  
ing treatment in the solution of protein and/or sugar,  
decrease of the antigen or antibody activity of immuno  
active substance caused during air drying procedure of the  
carrier can be prevented effectively.

10 The stabilized immuno active substances immobilized  
on a carrier is useful as a reagent for measuring physiolog-  
ically active substances in RIA or EIA.

Typical measuring systems in solid phase RIA and  
EIA are a competitive method and a sandwich method.

15 The competitive method is based on the competitive  
reaction between an unknown amount of the antigen in a  
test sample and known amount of the same radioisotopically  
or enzymatically labelled antigen to its antibody immobiliz-  
ed on the solid phase. Amount of the antigen in a test  
20 sample is quantified by measuring the solid phase bound or  
unbound amount of radioactivity or enzymatic activity of  
the labelled antigen.

On the other hand, the sandwich method is based  
on the reaction that two specific antibodies sandwich an  
25 unknown antigen to be measured. One of the antibodies is  
immobilized onto a solid phase and the other is labelled  
by a radioisotope or an enzyme. The amount of the antigen  
to be measured is quantified by measuring the bound amount

1 of radioactivity or enzymatic activity of antibody on the solid phase.

Needless to say, the application of the present invention is not limited to the typical measuring systems  
5 in RIA and EIA mentioned above. It also can be applied to various modified systems which utilize the immuno active substance immobilized on a carrier.

This invention is illustrated in detail by way of the following Examples, wherein all percents are by weight  
10 unless otherwise specified.

#### Reference Example 1

##### (1) Preparation of Antiinsulin Antibody-Bound Glass Beads

Commercially available glass beads (6 - 7 mm in diameter) (500 pieces) were washed with purified water,  
15 followed by washing with acetone. Then the glass beads were immersed in a 2%  $\gamma$ -aminotriethoxysilane/acetone solution and stood for 3 hours at room temperature. After the reaction, the glass beads were washed with acetone and purified water successively. The amino group-containing glass beads  
20 thus obtained were activated by immersing them in a 25% glutaraldehyde solution for 2 hours at room temperature. After extensively washed with purified water, the glass beads were immersed in 100 ml of 0.02 M phosphate buffer (pH 7.3) containing 3 mg of guinea pig antiinsulin antibody  
25 and allowed to stand at 4°C for 16 hours to bind the anti-insulin antibody to the glass beads. After the coupling reaction, the glass beads were washed with a 0.02 M phosphate

1 buffer (pH 7.3), and stored in a phosphate buffer (pH 7.3) containing 0.15M NaCl, 1% bovine serum albumin, 1 mM EDTA (ethylenediaminetetraacetic acid) and 0.05% NaN<sub>3</sub> in a cold place until the use.

5 (2) Preparation of Anti- $\beta_2$ -microglobulin Antibody-Bound Polystyrene Beads

Commercially available polystyrene beads (6.5 mm in diameter) (500 pieces) were washed with a 0.02 M phosphate buffer (pH 7.5) and then immersed in 100 ml of a 0.02 M  
10 phosphate buffer (pH 7.5) containing 3 mg of rabbit anti- $\beta_2$ -microglobulin antibody and allowed to stand at 4°C for 16 hours to bind the anti- $\beta_2$ -microglobulin antibody to the polystyrene beads. After the reaction, the polystyrene beads were washed with a 0.02 M phosphate buffer (pH 7.3), stored  
15 in a 0.02 M phosphate buffer (pH 7.3) containing 0.15 M NaCl, 1% bovine serum albumin, 1 mM EDTA and 0.05% NaN<sub>3</sub> in a cold place until the use.

(3) Preparation of Anti-C-Reactive Protein (C-RP) Antibody-Bound Poly(vinyl chloride) Plates

20 To each well of commercially available poly(vinyl chloride) microplates (U type, 96 wells), 0.1 ml of a 0.05 M carbonate buffer (pH 9.6) containing 5  $\mu$ g of mouse anti-C-RP antibody was added and allowed to stand at 4°C for 21 hours to bind the anti-C-RP antibody to the microplates. After  
25 the reaction, each well was washed with a 0.01 M phosphate buffer (pH 7.4) containing 0.05% polyoxyethylene sorbitan monolauryl ether (Tween 20, a trade name, manufactured by Kao-Atlas Co., Ltd.), added with 0.2 ml of a 0.01 M phosphate

1 buffer (pH 7.4) containing 1% bovine serum albumin, allowed  
to stand at 4°C for 19 hours, and stored in a cold place  
until the use.

(4) Preparation of Anti-CEA Antibody-Bound Glass Beads

5               Commercially available 500 glass beads (6 - 7 mm  
in diameter) were washed with purified water, followed by  
washing with acetone. Then the glass beads were immersed  
in a 2%  $\gamma$ -aminotriethoxysilane/acetone solution and stood  
for 3 hours at room temperature. After the reaction, the  
10 glass beads were washed with acetone and purified water  
successively. The glass beads thus obtained were activated  
by immersing in a 25% glutaraldehyde solution for 2 hours  
at room temperature. After extensively washed with purified  
water, the glass beads were immersed in 100 ml of 0.02 M  
15 phosphate buffer (pH 7.3) containing 3 mg of rabbit antibody  
and stood for 16 hours at 4°C to bind the anti CEA antibody  
to the glass beads. After the coupling reaction, the glass  
beads were washed with 0.02 M phosphate buffer (pH 7.3)  
and stored in 0.02 M phosphate buffer (pH 7.3) containing  
20 0.15 M NaCl, 1% bovine serum albumin, 1 mM EDTA and 0.05%  
 $\text{NaN}_3$  in a cold place until the use.

Reference Example 2

[1] Measurement of Insulin by EIA Method Using Antiinsulin  
Antibody-Bound Glass Beads

25 Measurement of Insulin by EIA

Reagents:

(1) Antiinsulin antibody-bound glass beads obtained in

1 Reference Example 1.

(2) Standard insulin of 0 to 320  $\mu$ U/ml

(3) Peroxidase labeled antiinsulin antibody.

(4) A 0.02 M phosphate buffer (pH 6.9) containing

5 0.15 M NaCl, 1% bovine serum albumin, 1 mM EDTA

and 0.1% sodium salicylate for diluting the above-mentioned reagents (2) and (3).

(5) 60 mg of o-phenylenediamine.

(6) 1.7 v/v% hydrogen peroxide solution.

10 (7) A 0.05 M citrate-0.1 M phosphate buffer (pH 4.8)

for dissolving the enzyme substrates of (5) and (6) mentioned above.

(8) 1.5 N  $H_2SO_4$ .

(9) A color developing reagent solution in an amount of

15 20 ml containing 60 mg of o-phenylenediamine and 200  $\mu$ l

of hydrogen peroxide obtained by dissolving the above-mentioned (5) and (6) in (7).

Assay Procedures:

To 500  $\mu$ l of the reagent (3) diluted with the  
20 reagent (4), 50  $\mu$ l of standard insulin solution was added,  
followed by addition of the reagent (1) to conduct the reaction at 37°C for 60 minutes. After the reaction, the beads were washed with 0.9% NaCl, followed by the addition of 500  $\mu$ l of the reagent (9) to start the enzymatic reaction.  
25 After incubating at 37°C for 15 minutes, 3.0 ml of the reagent (8) was added to stop the reaction and absorbance of the reaction mixture was measured at 492 nm.

- 1 [2] Measurement of  $\beta_2$ -Microglobulin by EIA Method Using  
Anti- $\beta_2$ -microglobulin Antibody-Bound Polystyrene Beads  
Measurement of  $\beta_2$ -Microglobulin by EIA

Reagents:

- 5 (1) Anti- $\beta_2$ -microglobulin antibody-bound polystyrene  
beads obtained in Reference Example 1.
- (2) Standard  $\beta_2$ -microglobulin of 0 to 200  $\mu\text{g}/\text{l}$ .
- (3) Peroxidase labeled anti- $\beta_2$ -microglobulin antibody.
- (4) A 0.02 M phosphate buffer (pH 6.9) containing
- 10 0.15 M NaCl, 1% bovine serum albumin, 1 mM EDTA,  
and 0.1% sodium salicylate for diluting the above-mention-  
ed reagents (2) and (3).
- (5) 60 mg of o-phenylenediamine.
- (6) 1.7 v/v% hydrogen peroxide solution.
- 15 (7) A 0.05 M citrate-0.1 M phosphate buffer (pH 4.8)  
for dissolving the enzyme substrates of (5) and (6) mentioned  
above.
- (8) 1.5 N  $\text{H}_2\text{SO}_4$ .
- (9) A color developing reagent solution in an amount
- 20 of 20 ml containing 60 mg of o-phenylenediamine and 200  $\mu\text{l}$  of  
hydrogen peroxide obtained by dissolving the above-mentioned  
(5) and (6) in (7).

Assay Procedures:

- To 1 ml of the reagent (3) diluted with the re-
- 25 agent (4), 20  $\mu\text{l}$  of standard  $\beta_2$ -microglobulin was added,  
followed by addition of the reagent (1) to conduct the  
reaction at 37°C for 60 minutes. After the reaction,



- 1 the beads were washed with 0.9% NaCl, followed by the  
addition of 500  $\mu$ l of the reagent (9) to start the enzymatic  
reaction. After incubating at 37°C for 15 minutes, 3.0 ml  
of the reagent (8) was added to stop the reaction and  
5 absorbance of the reaction mixture was measured at 492 nm.

[3] Measurement of C-RP by EIA Method Using Anti-C-RP  
Antibody-Bound Poly(vinyl chloride) Plates

Measurement of C-RP by EIA

Reagents:

- 10 (1) Anti-C-RP antibody-bound poly(vinyl chloride) plates  
obtained in Reference Example 1.
- (2) Standard C-RP of 0 to 1000 ng/ml.
- (3) Peroxidase labeled anti-C-RP antibody.
- (4) A 0.02 M phosphate buffer (pH 7.3) containing 1%  
15 bovine serum albumin, 0.5% polyoxyethylene nonylphenyl  
ether (Nonipol 300, a trade name, manufactured by Sanyo  
Chemical Industries, Ltd.) and 0.9% NaCl for diluting  
the above-mentioned reagents (2) and (3).
- (5) 60 mg of o-phenylenediamine.
- 20 (6) 1.7 v/v% hydrogen peroxide solution.
- (7) A 0.05 M citrate-0.1 M phosphate buffer (pH 4.8)  
for dissolving the enzyme substrates of (5) and (6) mentioned  
above.
- (8) 6N H<sub>2</sub>SO<sub>4</sub>.
- 25 (9) A color developing reagent solution in an amount  
of 20 ml containing 60 mg of o-phenylenediamine and 200  $\mu$ l  
of hydrogen peroxide obtained by dissolving the above-

1 mentioned (5) and (6) in (7).

Assay Procedures:

To each well, 100  $\mu$ l of standard C-RP diluted  
with the reagent (4) was added and allowed to stand at 37°C  
5 for 120 minutes. Then, the reaction solution was removed  
by suction and each well was washed with the reagent (4)  
extensively. After adding 100  $\mu$ l of the reagent (3), the  
reaction was conducted at 37°C for 120 minutes. After the  
reaction, each well was washed with the reagent (4), followed  
10 by addition of 100  $\mu$ l of the reagent (9) to start the  
enzymatic reaction. After incubating at room temperature for  
15 minutes, 50  $\mu$ l of the reagent (8) was added to stop the  
reaction and absorbance of the reaction mixture was measured  
at 490 nm by using a colorimeter for microplates.

15 [4] Measurement of CEA by EIA

Reagents:

- (1) Anti CEA antibody-bound glass beads obtained in  
Reference Example 1.
- (2) Standard CEA of 60  $\mu$ g/ml.
- 20 (3) Peroxidase labeled anti CEA antibody.
- (4) A 0.02 M phosphate buffer (pH 7.0) containing 0.15 M  
NaCl, 1% bovine serum albumin, 1 mM EDTA and 0.1% sodium  
salicylate for diluting the above-mentioned reagents (2)  
and (3).
- 25 (5) 60 mg of o-phenylenediamine.
- (6) 1.7 v/v% hydrogen peroxide solution

1     (7)       A 0.05 M citrate-0.1 M phosphate buffer (pH 4.8)  
for dissolving the enzyme substrates of (5) and (6) mentioned above.

      (8)       1.5 N  $\text{H}_2\text{SO}_4$ .

5     (9)       A color developing reagent solution in an amount  
of 20 ml containing 60 mg of o-phenylenediamine and 200  $\mu\text{l}$   
of 1.7% hydrogen peroxide obtained by dissolving the above  
mentioned (5) and (6) in (7).

Assay procedures:

      To 500  $\mu\text{l}$  of the reagent (3) diluted with the  
10 reagent (4), 50  $\mu\text{l}$  of standard CEA solution was added, followed by addition of the reagent (1) to conduct the reaction at 37°C for 18 hours. After the reaction, the beads were washed with 0.9% NaCl followed by the addition of 500  $\mu\text{l}$  of the reagent (9) to start the enzymatic reaction. After  
15 incubating at 37°C for 30 minutes, 3 ml of the reagent (8) was added to stop the reaction and absorbance of the reaction mixture was measured at 492 nm.

Example 1

Stabilization of Antiinsulin Antibody-Bound Glass Beads

20       After washing the antiinsulin antibody-bound glass beads prepared in Reference Example 1 with purified water, the glass beads were immersed in the following treating solutions (a) to (e) at room temperature for 30 to 40 minutes.

25     (a)       A 0.02 M phosphate buffer (pH 6.9) containing

1 5 w/v% sucrose and 1% bovine serum albumin.

(b) A 0.02 M phosphate buffer (pH 6.9) containing  
5 w/v% sucrose.

(c) A 0.02 M phosphate buffer (pH 6.9) containing  
5 1% bovine serum albumin.

(d) A 0.02 M phosphate buffer (pH 6.9) containing 1%  
water-soluble gelatin.

(e) A 0.02 M phosphate buffer (pH 6.9).

After the treatment, the glass beads were air dried  
10 at room temperature.

The glass beads thus obtained were subjected to  
a severe test by storing the glass beads in a constant  
temperature chamber at 40°C. Stability of the antibody-bound  
glass beads were evaluated as follows. A sample containing  
15 320  $\mu$ U/ml of insulin was measured by EIA method described  
in Reference Example 2 and stability of the glass beads  
was evaluated in terms of activity retention rate (%)  
compared with the measured value obtained by using control  
glass beads. The control glass beads were prepared as  
20 described in Reference Example 1 and stored at 4°C in the  
immersed state.

The results were shown in Table 1.

Table 1

Treating solution	Activity retention rate (%)	
	Stored for 2 weeks	Stored for 4 weeks
Control	100	100
(a)	94	89
(b)	70	68
(c)	47	19
(d)	71	66
(e)	14	9

1 Example 2

Stabilization of Anti- $\beta_2$ -microglobulin Antibody-Bound Polystyrene Beads

The anti- $\beta_2$ -microglobulin antibody-bound polystyrene  
5 beads prepared in Reference Example 1 were immersed in purified water. After removing water on a filter paper, the polystyrene beads were immersed in the following treating solutions (a) to (e) at room temperature for 30 to 40 minutes.

(a) A 0.02 M phosphate buffer (pH 6.9) containing  
10 5 w/v% sucrose and 1% bovine serum albumin.

(b) A 0.02 M phosphate buffer (pH 6.9) containing  
5 w/v% sucrose.

(c) A 0.02 M phosphate buffer (pH 6.9) containing  
1% bovine serum albumin.

15 (d) A 0.02 M phosphate buffer (pH 6.9) containing

1 1% water-soluble gelatin.

(e) A 0.02 M phosphate buffer (pH 6.9).

After the treatment, the polystyrene beads were taken out from the solutions and placed on a filter paper  
5 to remove the water and air dried at room temperature.

The polystyrene beads thus treated were subjected to the severe test in the same manner as described in Example 1 by storing them in the constant temperature chamber at 40°C. Stability of the antibody-bound polystyrene beads were  
10 evaluated as follows. A sample containing 200 µg/l of  $\beta_2$ -microglobulin was measured by EIA method described in Reference Example 2 and evaluated in terms of activity retention rate (%) compared with the measured value obtained by using control polystyrene beads. The control polystyrene  
15 beads were prepared as described in Reference Example 1 and stored at 4°C in the immersed state.

The results were shown in Table 2.

Table 2

Treating solution	Activity retention rate (%)	
	Stored for 2 weeks	Stored for 4 weeks
Control	100	100
(a)	102	101
(b)	93	81
(c)	81	42
(d)	80	79
(e)	42	37

1 Example 3

Stabilization of Anti-CRP Antibody-Bound Poly(vinyl chloride) Microplates

The anti-CRP antibody-bound poly(vinyl chloride) microplates prepared in Reference Example 1 were treated by using the following solutions and procedures.

(a) 4% Lactose solution was poured into each well of microplates and then each well was dried.

(b) 0.02 M Hepes buffer (pH 7.0) containing 1% bovine serum albumin was poured in each well of microplates and then each well was dried.

(c) 0.01 M Phosphate buffer (pH 7.4) containing 1% bovine serum albumin was poured in each well of microplates and stored in the poured state.

(d) Each well was air dried without treatment.



1           After the treatment, microplates were stored at  
25°C for 7 weeks. Stability of the antibody-bound micro-  
plates was evaluated as follows. A sample containing 1000  
µg/ml CRP was measured by EIA method described in Reference  
5 Example 2 and evaluated in terms of activity retention rate  
(%) compared with the measured value obtained by control  
microplates which were prepared in the same manner as  
described in Reference Example 1 at the time of use.

The results were shown in Table 3.

Table 3

Treating procedures	Activity retention rate (%)
Control	100
(a)	95
(b)	90
(c)	89
(d)	0

10 Example 4

After washing the anti CEA antibody-bound glass  
beads prepared in Reference Example 1 with purified water,  
the glass beads were immersed in the following solutions  
(a) to (h) at room temperature for 30 to 40 minutes.

15       (a)     A 0.02 M phosphate buffer (pH 7.0) containing



1 5% sucrose and 1% bovine serum albumin

(b) A 0.02 M phosphate buffer (pH 7.0) containing  
5% lactose.

(c) A 0.02 M tris-HCl buffer (pH 7.2) containing  
5 5% mannose and 1.5% water-soluble gelatine.

(d) A 0.02 M tris-HCl buffer (pH 7.2) containing 4%  
dextrin.

(e) A 0.02 M Hepes buffer (pH 7.2) containing 5%  
sucrose.

10 (f) A 0.02 M phosphate buffer (pH 7.0).

(g) A 0.02 M tris-HCl buffer (pH 7.2).

(h) A 0.02 M Hepes buffer (pH 7.2).

After the treatment, the glass beads were air  
dried at room temperature.

The glass beads thus obtained were subjected to  
15 a severe test by storing them in a constant chamber at 40°C.  
Stability of the antibody-bound glass beads were evaluated  
as follows. A sample containing 60 µg/ml CEA was measured by  
EIA method described in Reference Example 2. The stability of  
the glass beads was evaluated in terms of activity retention  
20 rate (%) compared with the measured value obtained by using  
control glass beads. The control glass beads were prepared  
as described in Reference Example 1 and stored at 4°C in the  
immersed state.

The results were shown in Table 4.

Table 4

Treating solution	Activity retention rate (%)	
	Stored for 2 weeks	Stored for 4 weeks
Control	100	100
(a)	98	92
(b)	75	70
(c)	92	85
(d)	70	65
(e)	83	76
(f)	15	6
(g)	12	3
(h)	20	11

CLAIMS:

1. A process for stabilizing an immuno active substance immobilized on a carrier, which comprises immersing a carrier bound an immuno active substance in a solution of at least one member selected from the group consisting of a sugar and a protein.
2. A process according to Claim 1, which further comprises drying the immersed carrier.
3. A process according to Claim 1, wherein the carrier is a synthetic polymer material or an inorganic substance.
4. A process according to Claim 1, wherein the immuno active substance is an antigen.
5. A process according to Claim 1, wherein the immuno active substance is an antibody.
6. A reagent for measuring a physiologically active substance comprising as a component an immuno active substance immobilized on a carrier and stabilized by immersing the carrier bound the immuno active substance in a solution of at least one member selected from the group consisting of a sugar and a protein.
7. A reagent according to Claim 6, wherein the carrier is an inorganic substance.
8. A reagent according to Claim 6, wherein the carrier is a synthetic polymer material.
9. A reagent according to Claim 6, wherein the immuno active substance is an antigen.
10. Use of a reagent according to Claim 6 for measuring a physiologically active substance by enzyme immunoassay

or radioimmunoassay.

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European Patent  
Office

# EUROPEAN SEARCH REPORT

0140489  
Application number

EP 84 30 5286

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P, X	GB-A-2 124 231 (CORNING GLASS WORKS) * Whole document *	1-10	G 01 N 33/543 G 01 N 33/545 G 01 N 33/551
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X	GB-A-2 016 687 (ABBOTT LABORATORIES) * Whole document *	1-10	
	---		
X	EP-A-0 063 810 (CIBA-GEIGY AG.) * Claims 1,2,7,32,33 *	1-10	
	---		
X	EP-A-0 042 755 (UNILEVER NV.) * Claims 1,4; page 10, lines 11-16 *	1-10	
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			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			G 01 N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 16-11-1984	Examiner GRIFFITH G.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	